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Regulatory and functional interactions between the somatic sex regulatory gene *transformer* and the germline genes *ovo* and *ovarian tumor*

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SUMMARY

In *Drosophila*, compatibility between the sexually differentiated state of the soma and the sex chromosome constitution of the germline is required for normal gametogenesis. In this study, we defined important aspects of the soma-germline interactions controlling early oogenesis. In particular, the sex-specific germline activity of the *ovarian tumor* promoter was found to be dependent upon somatic factors controlled by the somatic sex differentiation gene *transformer*. This regulation defines whether there is sufficient *ovarian tumor* expression in adult *XX* germ cells to support oogenesis. In addition, the *ovarian tumor* function required for female germline differentiation is dependent on the activity of another germline gene, *ovo*, whose regulation is *transformer*-independent. These and other data indicate that *ovarian tumor* plays a central role in coordinating regulatory inputs from the soma (as regulated by *transformer*) with those from the germline (involving *ovo*). We also demonstrate that *transformer*-dependent interactions influence whether *XX* germ cells require *ovarian tumor* or *ovo* functions to undergo early gametogenic differentiation. These results are incorporated into a model hypothesizing that the functions of *ovarian tumor* and *ovo* are dependent on an early sex determination decision in the *XX* germline that is at least partially controlled by somatic *transformer* activity.

Key words: Oogenesis, Germline, *ovarian tumor*, *transformer*, *ovo*, *Drosophila*

INTRODUCTION

In *Drosophila melanogaster*, the sexual differentiation of the germline requires a complex interplay between cell autonomous factors controlled by the X:A ratio of the germ cells and sex-specific somatic functions (Nöthiger et al., 1989; Steinmann-Zwicky et al., 1989; Steinmann-Zwicky, 1994a,b; Nagoshi et al., 1995). For example, certain allele combinations of *transformer*, *transformer-2* and *doublesex* can cause chromosomally female (*XX*) flies to develop with most of their somatic tissues having a male identity, i.e., '*XX* pseudomales' (Sturtevant, 1945; Watanabe, 1975; Nöthiger et al., 1980). In these flies, oogenesis is aborted and there is even occasionally what appears to be early spermatogenic development (Sturtevant, 1945; Seidel, 1963; Watanabe, 1975; Nöthiger et al., 1980, 1987, 1989). Since the germline expressions of these sex regulatory genes are not required for early stages of gametogenesis (Marsh and Wieschaus, 1978; Schüpbach, 1982), the aberrant germline phenotypes must result from the male transformation of the soma.

The interpretation of these studies is complicated by the difficulty in diagnosing the differentiated state of abnormal germ cells when using only morphological criteria. Therefore, we took advantage of molecular probes that facilitated the identification of sex- and stage-specific germline structures. These characteristics are described as follows. In the most apical portion of the adult germarium (region 1), germline stem cells divide asymmetrically to produce daughter stem cells and cystoblasts. These two cell types are identified by the presence of a globular spectrosome which can be detected with antibodies and reagents specific for spectrin, a product from the *hu-li tai shao* (*hts*) gene (an adducin-like protein), and f-actin (Lin et al., 1994; Rodesch et al., 1997). The cystoblast undergoes a set of four mitotic divisions noted by incomplete cytokinesis, to produce a 16-cell cyst connected by cytoplasmic bridges (ring canals) through which passes a branched, proteinaceous structure called the fusome (Lin et al., 1994; Lin and Spradling, 1995). The fusome results from the elongation and branching of the spectrosome during the cystocyte divisions, a transition associated with decreased levels of fusome f-actin (Rodesch et al., 1997). Shortly after the completion of the cystocyte divisions, the somatic follicle cells begin to associate with the 16-cell syncytium. This marks the beginning of germarial region 2, during which the fusome disappears and f-actin and another *hts* product (HTS-RC) integrates into the ring canals (Lin et al., 1994; Robinson et al., 1994). In region 3, the germline cyst is surrounded by follicle cells to form a stage 1 egg chamber. This period is noted by the localization of a product from the *kelch* gene to the ring canals (Xue and Cooley, 1993; Robinson et al., 1994). These events differ from that seen in spermatogenesis in two aspects relevant to our study. First, the multibranched spermatogenic fusome contains substantial levels of f-actin and

persists past the 16-cell stage into the meiotic divisions (Hime et al., 1996). Second, male germline ring canals do not incorporate f-actin, HTS-RC or KELCH proteins, indicating a different maturation process (Hime et al., 1996). Therefore, these components of spectrosomes, fusomes and ring canals provide important tools to identify the differentiated state of germ cells under different mutant conditions.

It is not clear which germline genes are influenced by the proposed somatic interactions. Three possible candidates based on their early and sex-specific roles in female germline differentiation are *ovarian tumor* (*otu*), *ovo* and *Sex-lethal* (*Sxl*). The expression of *otu* is required in the germline at several stages, if not continually, in oogenesis (reviewed in King and Storto, 1988). The null mutant phenotype is characterized by the absence of egg chambers in an otherwise normal ovary, denoted as the quiescent phenotype (King and Riley, 1982), although substantial numbers of germ cells are still present in the germarium (Rodesch et al., 1995). Null and severe loss-of-function mutations can also produce 'ovarian tumors,' a phenotype characterized by egg chambers containing hundreds of seemingly undifferentiated germ cells. Both the quiescent and tumorous cells are aborted at early oogenic stages, during the cystocyte divisions prior to cyst formation (Rodesch et al., 1997). Mutations in *otu* have no significant effect on spermatogenesis, although some aberrations in male courtship behavior have been reported (Tirronen et al., 1993).

The *ovo* gene has been implicated in regulating sex determination and dosage compensation in the germline (Oliver et al., 1990, 1993, 1994; Wei et al., 1994). This is based primarily on observations that *ovo* null *XX* germ cells are typically not found in the adult ovary, presumably because of reduced cell viability (Oliver et al., 1987, 1990; Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). In addition, certain *ovo* allele combinations produce tumorous germ cells which morphologically resemble primary spermatocytes (Oliver et al., 1987, 1990). These phenotypes make *ovo* a candidate target for a somatic signal regulating early oogenesis, although the expression of *ovo* in adult germ cells does not appear to be responsive to somatic influences (Oliver et al., 1994). Interestingly, there is recent evidence that *ovo* might directly regulate *otu* (Hager and Cline, 1997; Lu et al., 1997). The OVO protein can bind to sites in the *otu* promoter, which displays sensitivity to changes in the dosage of ovo^+ function. It is not known when this putative regulation of *otu* occurs nor what role it plays in oogenesis.

Besides its essential role in the female germline, the *Sxl* gene controls sex determination and dosage compensation in somatic cells (reviewed in Cline and Meyer, 1996). Female-sterile alleles represent hypomorphic mutations that specifically disrupt the germline *Sxl* function but allow normal somatic development (Perrimon et al., 1986; Salz et al., 1987). These result in ovarian tumors superficially similar to those produced by *otu* and *ovo* (reviewed in Pauli and Mahowald, 1990; Steinmann-Zwicky, 1992). Pole cell transplantation studies have led to the suggestion that the soma might influence aspects of germline sex determination through the regulation of *Sxl*. (Steinmann-Zwicky, 1994b).

With respect to interactions with the germline, *transformer* (*tra*) is the most extensively studied of the somatic sex regulatory genes. The masculinization of *XX* soma due to loss-of-function *tra* mutations cause germ cell aberrations during first instar larval stages (Seidel, 1963; Steinmann-Zwicky, 1994a) and misregulated sex-specific germline gene expression in the embryo (Staab et al., 1996). Furthermore, when *XY* soma is feminized by ectopic *tra* expression (to form 'pseudofemales,' McKeown et al., 1988), the somatic components of the ovaries are sufficiently 'female' that they can support the maturation of transplanted *XX* germ cells (Steinmann-Zwicky, 1994a). The pseudofemale soma also appears to partially feminize the XY germline, as these cells now required the normally female-specific *otu* function for optimal proliferation (Nagoshi et al., 1995). These observations indicate that *tra* controls a substantial portion of the somatic-germline interactions affecting early gametogenic differentiation.

In this paper, we tested for regulatory and functional interactions between the somatically required *tra* gene and the germline functions of *otu* and *ovo*. We found that *tra* function determines whether sufficient *otu* is expressed in adult *XX* germ cells to support oogenesis. This regulation occurs at the level of the *otu* promoter, and imposes stage- and sex-specific expression of *otu* that differs significantly from the pattern of *ovo* promoter activity. We show that *otu* has two functions, one regulating oogenic differentiation that is dependent on *ovo*, and one influencing germ cell proliferation that appears to be *ovo*-independent. These and other observations indicate *otu* plays a critical role in linking the somatic sex regulatory pathway with the germline genes controlling early oogenesis. Finally, we present evidence that the germline requirement for *ovo* and *otu* are influenced by *tra*-dependent somatic interactions. These data give rise to a new hypothesis for the regulation of early oogenesis that differs significantly from current models.

MATERIALS AND METHODS

Fly strains and crosses

Flies were raised on standard cornmeal, molasses, yeast, agar media containing propionic acid as a mold inhibitor and supplemented with live yeast. Unless otherwise noted, alleles and chromosomes used are described in Lindsley and Zimm (1992). The *hs-otu* strain was obtained from the laboratory of P. Geyer. The construct consists of a 4 kb *Hpa*I *otu* genomic fragment starting 64 bp downstream of the transcription start site and ending 139 bp downstream of the translation stop codon, under the regulation of the *Drosophila hsp70* promoter and the polyadenylation sequences of the α-tubulin gene. The transgene is marked with ry^+ and is inserted into an unmarked second chromosome. The *otu-lacZ* strain contains the w^+ marked transgene inserted on the second chromosome (Rodesch et al., 1995). To construct *pOtu104*, a 0.4 kb *Hpa*I-*Pst*I fragment containing the *otu* polyadenylation site was inserted into the equivalent sites in the w^+ *Drosophila* transformation vector *Casper-3* (Pirrotta, 1988), to form *Casper-3′-otu*. This was followed by the insertion of a 736 bp *Eco*RV(artificial site)-*Hpa*I *otu* promoter fragment, containing 672 bp of sequence upstream of the primary transcription start site, into the *Hpa*I site of the *Casper-3′-otu* subclone. *Csp-3-8* represents the orientation in which the *otu* promoter and poly-adenylation sequences are now separated by a single *Hpa*I site. Finally, a cDNA specific for the 104 kDa *otu* isoform was inserted in the appropriate orientation into the *Csp-3-8 Hpa*I site. Germline transformation of *pOtu104* gene was carried out by standard methods (Rubin and Spradling, 1982).

Construction of *XX* pseudomales

XX flies that carry mutations in *transformer* develop as somatic males

(denoted as *XX* pseudomales). The heterozygous combinations of two loss-of-function alleles, tra^1 and tra^4, were used to optimize the isolation of viable pseudomales. Chromosome abbreviations: tra^4 = tra^4 kar^2 ry^5 *red*, $otu^{P\Delta1}$ = $otu^{P\Delta1}$ *v f*, ovo^{D1rS1} = *y w* ovo^{D1rS1}, Sxl^{f5} = *y* Sxl^{f5}. *XX* pseudomales wild type or mutant for *ovo*, *otu* or *Sxl* were produced by the following crosses: (a)+/+; tra^1/TM6 × *FM6/Y*; tra^4/*TM6*, (b)+/+; tra^4/*TM6* × +/B^sY; tra^1/*TM6*, (c) $otu^{P\Delta1}$/*FM6*; tra^1/TM6 × $otu^{P\Delta1}$/B^sY; tra^4/*TM6*, (d) ovo^{D1rS1}/*FM6*; tra^1/TM6 × ovo^{D1rS1}/B^sY; tra^4/*TM6*, (e) Sxl^{f5}/*FM6*; tra^1/TM6 × Sxl^{f5}/B^sY; tra^4/*TM6*. Pseudomales carrying *hs-otu* were produced by (g) *hs-otu/hs-otu*; tra^4/*TM6* × +/B^sY; tra^1/*TM6*, (h) ovo^{D1rS1}/*FM6*; *hs-otu/hs-otu*; tra^4/*TM6* × ovo^{D1rS1}/B^sY; tra^1/*TM6*, (i) *hs-otu/hs-otu*; tra^4/*TM6* × +/B^sY; *otu-lacZ/CyO*; tra^1/*TM6*, (j) *hs-otu/hs-otu*; tra^4/*TM6* × +/B^sY; *ovo-lacZ/CyO*; tra^1/*TM6*, (k) *hs-otu/hs-otu*; tra^4/*TM6* × +/B^sY; *BC69/CyO*; tra^1/*TM6*. Pseudomales carrying *pOtu104* were produced by (l) *pOtu104/pOtu104*; tra^4/*TM6* × +/B^sY; tra^1/*TM6*, (m) $otu^{P\Delta1}$/*FM6*; *pOtu104/pOtu104*; tra^4/*TM6* × $otu^{P\Delta1}$/B^sY; tra^1/*TM6*. All crosses were cultured at either room temperature (20-23°C) or in a 25°C incubator. Both conditions lead to identical phenotypes (data not shown).

Construction of *XY* pseudofemales

The *hs-tra* transgene places *tra* under the control of the *Drosophila hsp70* promoter (McKeown et al., 1988; Rodesch et al., 1997; McKeown et al., 1988). To create *ovo* mutant *XY* pseudofemales, *y w* ovo^{D1rS1}*/FM6; Ki hs-tra* p^p *Df(tra)/TM6* females were crossed to +/B^sY males. *ovo* mutant *XY* pseudofemales were identified as white, Bar-eyed females.

Whole-mount immunohistochemistry and fluorescent labeling

The differentiated state of mutant and wild-type germ cells was determined by immunohistochemical analyses using antibodies and reagents specific for spectrosomes, fusomes and ring canals. Adults were aged for 4-7 days (unless otherwise noted) after eclosion. Gonads were dissected in PBS (130 mM NaCl, 7 mM Na_2HPO_4-$2H_2O$, 3 mM NaH_2PO_4-$2H_2O$). The tissues were fixed in a 1:1 solution of fix:heptane (fix: 4% paraformaldehyde in PBS) for 20 minutes with gentle agitation. Tissues were washed 4× in PBT (0.1% Triton X-100, 0.05% Tween 80 in PBS) for 15 minutes. These were then permeabilized for at least 2 hours in blocking buffer (PBT + 1 mg/ml crystalline bovine serum albumin, Sigma) at room temperature.

All antibodies were diluted to the appropriate concentration in blocking buffer. Incubations with primary antibodies were performed at 4°C overnight. Primaries included the following monoclonals: anti-alpha-spectrin (3A9, 1:200 dilution; from D. Branton), anti-HTS-RC (HTS 655 4C, 1:100) and anti-KELCH (1B, 1:4), both from L. Cooley, and a polyclonal VASA antibody preparation (1:1000) from L. and Y. Jan. Primaries were removed with three 15 minute washes in PBT, followed by incubation with secondary antibodies (diluted 1:200) for 3 hours at room temperature. Secondaries used were biotinylated anti-mouse and anti-rabbit IgG (Sigma), and Oregon Green or Texas Red-conjugated anti-mouse IgG (Molecular Probes). With biotinylated secondaries, the preparations were fluorescently labeled using Texas Red or Oregon Green-conjugated streptavidin reagents (Molecular Probes) diluted 1:500 in blocking buffer and incubated for 30 minutes at room temperature. Phalloidin staining was achieved by dissolving 2 units of either Texas Red or Oregon Green-conjugated phalloidin (Molecular Probes) in 200 μl blocking buffer and incubating for 30 minutes at room temperature. Preparations were subsequently washed 3× for 15 minutes in PBT. Nuclei were labeled by performing the final antibody rinses in 0.2 μg/ml DAPI in PBT. Preparations were mounted in Vectashield (Vector Laboratories).

β-galactosidase staining

Construction and properties of the *otu-lacZ* construct are described in Rodesch et al. (1995). *BC69* is an enhancer trap line under the control of the germline-specific *vasa* promoter and was a gift from F. Laski (Rodesch et al., 1995; Heller and Steinmann-Zwicky, 1998). *ovo-lacZ* places β-galactosidase under the control of the *ovo* promoter (Mèvel-Ninio et al., 1995).

Gonads were dissected in PBS, then incubated in 50% fixative: 50% heptane in a covered depression slide with agitation for 3 minutes. The tissue was rinsed 3× in PBS + 0.1% Triton X-100. The tissue was incubated in staining solution overnight at room temperature in the dark. After staining, the preparation was washed 5× for 20 minutes with PBS. The tissue was mounted in 50% glycerol in PBS. Stock solutions: solution A, 6.75 g/l NaCl, 6.63 g/l KCl, 0.66 g/l $MgSO_4 \cdot 7H_2O$, 0.54 g/l $MgCl_2 \cdot 6H_2O$; 0.33 g/l $CaCl_2 \cdot 2H_2O$; solution B, 1.4 g/l Na_2HPO_4, 0.1 g/l KH_2PO_4, taken to pH 7 with NaOH; and solution C, the same as solution A but with 3.7% formaldehyde. Fixative: 9 parts solution C and 10 parts solution B. Staining solution: 0.75 mls of a mixture of 9 parts solution A and 10 parts solution B, 0.1 ml 50 mM potassium ferricyanate, 0.1 ml 50 mM potassium ferrocyanate, 50 μl 100 μg/μl 5-bromo-4-chloro-3-indoxyl-β-galactopyranoside (X-gal) in N, M′-dimethylformamide, to a total volume of 1.0 ml in water.

Microscopy

Confocal images were obtained on a Nikon Optiphot using a Bio-Rad MRC 1024 confocal laser apparatus. Sections were manipulated using Bio-Rad Lasersharp image analysis software and transferred to Adobe Photoshop for figures. Other microscopy was performed on an Olympus Vanox AHBT3 microscope using an Optronics LX450A camera for image capture.

RESULTS

Fusome and ring canal phenotypes of *XX* pseudomale germ cells

XX pseudomales produced by *tra* mutations contain three types of gonads that differ in the viability and sexual phenotype of their germ cells (Brown and King, 1961; Seidel et al., 1963; Nöthiger et al., 1989). We repeated this analysis using molecular probes that provided more definitive criteria for categorizing the germline phenotypes. Gonads from ‘normal’ *XX* pseudomales (i.e., carrying wild-type alleles of *ovo*, *otu* and *Sxl*) were simultaneously labeled with antibodies for spectrin and HTS-RC to identify spectrosomes, fusomes and oogenic ring canals. Three categories of gonads were defined.

The most common class were the ‘atrophic’ gonads, found in about half of the pseudomales examined (39/80; Fig. 1A). These contained no differentiating germ cells, as spectrosomes, fusomes, or ring canals were not observed. Confirmation of this interpretation came from a separate experiment in which gonads of this type were double-labeled with antibodies for the germline-specific VASA protein (Hay et al., 1988) and spectrin. In every case examined (n=10), gonads lacking cells with spectrosomes and fusomes also failed to label with VASA (Fig. 2A).

The next two categories were composed of gonads with germ cells initiating gametogenic differentiation, as demonstrated by the presence of spectrosomes and fusomes (Fig. 2B). These were subdivided depending on whether they expressed the HTS-RC protein, an oogenic characteristic. Gonads without HTS-RC-positive cells were designated as ‘non-oogenic’ and were found in 19% (15/80) of pseudomales (Fig. 1A). These could derive from aborted oogenesis before HTS-RC expression or if the germ cells underwent male

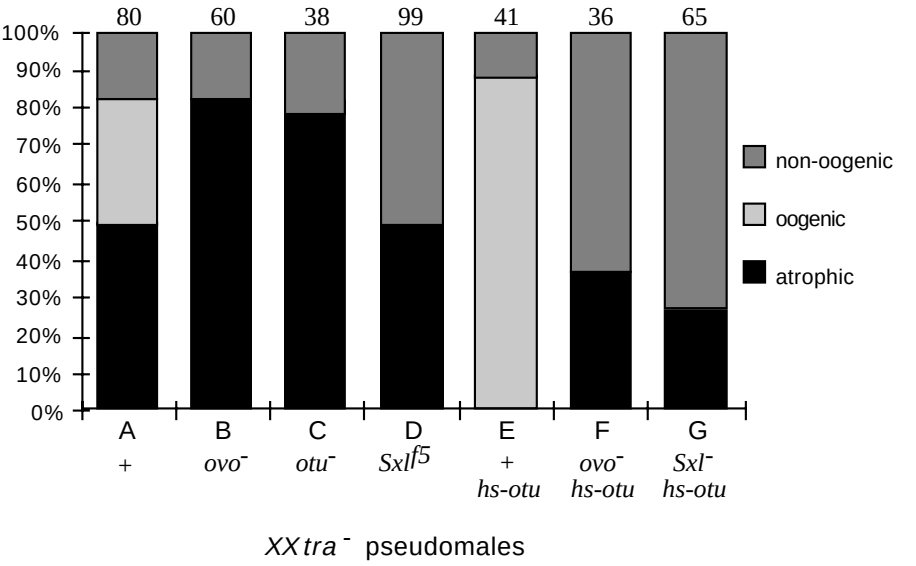

Fig. 1. Distribution of *XX* pseudomale gonad types produced by mutations in *ovo*, *otu* and *Sxl*. *tra* mutant *XX* gonads were labeled with antibodies specific for VASA and HTS-RC. Atrophic gonads are VASA-negative. Non-oogenic gonads have VASA-positive germ cells that do not express HTS-RC in their ring canals. Oogenic gonads are VASA-positive and HTS-RC-positive. Number at the top of each column indicates the number of gonads examined for that genotype. All genotypes are *XX;; tra*^{4} *kar*^{2} *ry*^{5} *red/tra*^{1}. Other mutations in each genotype: (A) *y w ovo*^{D1rS1}*/FM6*. (B) *y w ovo*^{D1rS1}*/y w ovo*^{D1rS1}. (C) *otu*$^{P\Delta1}$ *v f/otu*$^{P\Delta1}$ *v f*. (D) *y Sxl*^{f5}*/y Sxl*^{f5}. (E) *y w ovo*^{D1rS1}*/FM6; hs-otu/CyO*. (F) *y w ovo*^{D1rS1}*/ y w ovo*^{D1rS1}*; hs-otu/CyO*. (G) *y Sxl*^{f5}*/y Sxl*^{f5}*; hs-otu/CyO*.

differentiation. It appears that the latter make up the majority of non-oogenic gonads in normal pseudomales, as germ cells of this class were typically connected by multibranched fusomes containing f-actin, a spermatogenic characteristic (Fig. 2C). The remaining 'oogenic' class was found in 33% (26/80) of *XX* pseudomales. These were characterized by one or more cell clusters expressing HTS-RC (Fig. 2D,E). In these gonads, the 'feminized' clusters were surrounded by cells without HTS-RC expression, indicating female germline differentiation was infrequent and sporadic (Fig. 2E).

ovo, otu and *Sxl* are only required in *XX* germ cells undergoing female differentiation

We next compared the effects of an *ovo* null mutation, *ovo*^{D1rS1}, on *XX* germ cells developing in pseudomale testes and female ovaries. In females, *ovo*^{D1rS1} mutant *XX* germ cells typically arrest beginning at larval gonial stages (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995, although see Oliver et al., 1990 for evidence of earlier arrest). Occasionally, mutant germ cells survived to the adult stage, where they were sufficiently viable to express the germline-specific VASA protein (Fig. 2F1; Rodesch et al., 1995). However, these cells generally failed to undergo gametogenic differentiation as seen by the absence of spectrosomes, fusomes or ring canals (Fig. 2F2; R. N. Nagoshi and S. H., unpublished data). We reasoned that, if the requirement for *ovo* is solely dependent on the X:A ratio, then the phenotype of *ovo* mutant germ cells in pseudomales should be at least as severe. In this case, the *ovo*^{D1rS1} mutant *XX* pseudomale gonads should be either atrophic or contain a few clusters of mostly undifferentiated germ cells. Such a result had previously been reported, though without supporting data (Steinmann-Zwicky, 1989).

Our studies showed a more complicated phenotype. Although there was an increase in the frequency of atrophic gonads (82%) compared to normal pseudomales (48%), we also found many of the non-oogenic type (11/60, Fig. 1B). The non-oogenic gonads contained VASA-positive germ cells (Fig. 2G), with spectrosomes and multibranched fusomes (Fig. 2H,I). This indicates that not only were a substantial fraction of the mutant germ cells viable in adults, but gametogenic differentiation was occurring as well. The frequency of the non-oogenic gonads in *ovo* mutant pseudomales was essentially unchanged from that observed in normal pseudomales (18% versus 19%). This suggests the observed increase in the atrophic category was due primarily to the loss of the oogenic class (Fig. 1B).

Mutations in *otu* gave results similar to that described for *ovo* (Fig. 1C). Again there was a reduction in oogenic gonads occurring concomitantly with a compensating increase in atrophic testes. As with both *ovo*$^{+}$ and *ovo* mutant pseudomales, approximately 20% of gonads were non-oogenic and these frequently contained multibranched fusomes. This suggests that *otu* and *ovo* mutations specifically disrupt only those germ cells attempting female differentiation, rather than the indiscriminate elimination of the entire *XX* germline. This possibility was supported by more detailed analysis of a sampling of the *ovo*$^{-}$ non-oogenic pseudomales. In all gonads examined (8/8), multibranched fusomes were present containing f-actin, a characteristic of early spermatogenesis (the same phenotype as that seen in Fig. 2C for non-oogenic *ovo*$^{+}$ pseudomales).

A different phenotype was observed in *XX* pseudomales mutant for the germline function of *Sxl*. As with *ovo* and *otu*, *Sxl* female-sterile mutations blocked oogenic differentiation in *XX* pseudomales (Fig. 1D). However, this did not lead to more atrophic gonads. Instead there was an increase in the non-oogenic category such that the frequency of non-atrophic gonads was equal to *Sxl*$^{+}$ pseudomales (compare Fig. 1A and D). These observations indicate a fundamental difference in the germline phenotype caused by this *Sxl* allele compared to *ovo* and *otu* mutations, and demonstrate that aborted oogenesis does not inevitably lead to reduced germ cell viability in pseudomales.

Increased *otu* expression can feminize *XX* pseudomale germ cells

The *hs-otu* construct places the *otu* gene under the control of the *Drosophila* heat-shock *hsp70* promoter. This construct can suppress all *otu* mutations to fertility at room temperature (Rodesch et al., 1995; data not shown). We previously showed that *hs-otu* could alter the *XX* pseudomale gonadal phenotype;

however, the nature of this change was not determined (Nagoshi et al., 1995). To examine whether and to what degree *otu* expression could induce oogenic development in pseudomales, immunohistochemical studies were performed. When continually cultured at 20-25°C, *hs-otu* pseudomale gonads were as much as two to three times longer than normal (Fig. 3A,B). In addition, 88% of the *hs-otu* gonads examined (36/41) showed extensive HTS-RC-labeling of ring canals (Figs 1E, 3C). These 'feminized' gonads displayed a developmental progression of gametogenic stages, which could be subdivided into three sections corresponding to germarial regions 1-3 in normal oogenesis (I-III, Fig. 3C).

Section I includes the most apical portion of the *hs-otu XX* pseudomale testis and was found to contain gametogenic stages characteristic of germarial region 1 in ovaries (Fig. 3D). Localized at the distal tip was a group of cells containing spectrosomes, a characteristic of germline stem cells and cystoblasts. Immediately adjacent were clusters of cells connected by branched fusomes containing spectrin.

In section II, the fusomes have disappeared and the differentiation of the ring canals continues with the deposition of HTS-RC and f-actin (Fig. 3E). The localization of these two components to the rings does not occur simultaneously. Clusters of germ cells were detected in which all the ring canals contained HTS-RC, but only a subset were labeled by phalloidin. In comparison, by the end of section II all the ring canals were found to contain both factors. This suggests sequential deposition in which HTS-RC is localized first to the ring canal, followed by f-actin. A similar sequence of events is thought to occur during normal oogenesis in germarial region 2 (Yue and Spradling, 1992).

In section III, the pseudomale germ cells have differentiated to postgermarial stages as defined by the expression of *kelch*. The KELCH protein is localized to female ring canals after the deposition of HTS-RC and f-actin (Robinson et al., 1994). It first becomes detected in female ring canals in stage 1 egg chambers, but is not seen in all ring canals until stage 4 (Robinson and Cooley, 1997). In *hs-otu XX* pseudomales, the germ cell clusters in section III contained thick ring canals with virtually all showing KELCH deposition along the inner surface of the f-actin layer (Fig. 3F). In comparison, no KELCH-labeled ring canals were observed in *XX* pseudomales without *hs-otu* (n=15), indicating that oogenesis was not only less frequent, but also more limited. Consistent with the more mature ring canal phenotype were the large germ cell nuclei that stained extensively with DAPI, characteristics suggestive of polyploidy (Fig. 3G). Frequently, clusters of large cells were associated with a smaller cell containing at least four ring canals. This phenotype suggests oocyte differentiation and nurse cell polyploidy, consistent with oogenic development to as late as stage 4.

Taken together, these results indicate the masculinizing effect of male soma (or the absence of female soma) on *XX* germ cells can be partially, but consistently, overridden by the expression of *otu* from a heterologous promoter. The resulting fusome and ring canal development follows the same sequence of events as occurs in normal oogenesis. Therefore, pseudomale germ cells are competent to both initiate and undergo substantial oogenesis if provided with adequate levels of *otu*.

ovo and *Sxl* are required for *otu*-induced oogenic differentiation in *XX* pseudomales

We next determined whether the *otu*-induced feminization of

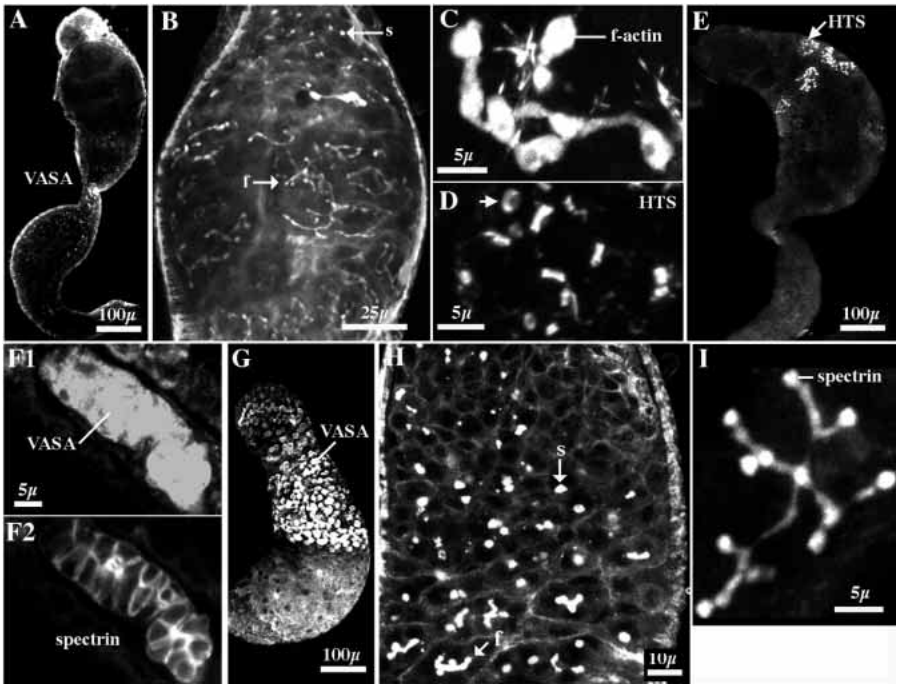

Fig. 2. Germ cell and gonadal phenotypes of ovo^{+} and ovo^{-} *XX* pseudomales. *tra* mutant *XX* pseudomale gonads were examined by immunohistochemical analysis for the presence of germ cells, fusomes, and ring canals. Nuclei were stained by DAPI. (A) Atrophic *XX* pseudomale testis (germarial region at top) labeled with anti-VASA. Gonads of this class are devoid of VASA-positive germ cells and do not have spectrosomes or fusomes (data not shown). (B) *XX* pseudomale non-oogenic gonad labeled with anti-spectrin. Spectrosomes (s) are found in stem cells and cystoblasts. Spectrosomes give rise to branched fusomes (f) that connect cystocyte clusters. (C) Typical fusome from non-oogenic *XX* pseudomale germ cells labeled with phalloidin. Presence of f-actin is characteristic of spermatogenic fusome. (D) HTS-RC-labeled ring canals from oogenic class *XX* pseudomale gonad (arrow). (E) Oogenic *XX* pseudomale testis labeled with anti-HTS-RC. Arrow points to one of five germ cell clusters with HTS-RC-positive ring canals. (F1) ovo^{D1rS1} mutant *XX* female ovariole labeled with anti-VASA antibodies. Large clusters of VASA-positive germ cells are present in some germaria. (F2) Same ovariole as (F1) labeled with anti-spectrin. Cell periphery is labeled but there are no spectrosomes or fusomes. (G) ovo^{D1rS1} mutant *XX* pseudomale testis of the non-oogenic class labeled with anti-VASA antibodies. Note clusters of VASA-positive germ cells in anterior half of testis. These do not express the female-specific HTS-RC protein. (H) Germarium of ovo^{D1rS1} mutant *XX* pseudomale testis labeled with anti-spectrin antibodies. Spectrosomes (s) and fusomes (f) are present. (I) A higher magnification of a highly branched fusome from an *ovo* mutant *XX* pseudomale gonad labeled with anti-spectrin. All plates except D are confocal images.

the *XX* pseudomale germline was dependent on *ovo* and *Sxl* function. *XX* pseudomales were constructed carrying one copy of *hs-otu* and either homozygous mutant for ovo^{D1rS1} or Sxl^{f5}. The mutant gonads were labeled for the germline-specific product, VASA, and the female ring canal protein, HTS-RC. In neither genotype was there an increased frequency of oogenic gonads due to *hs-otu*, indicating that *ovo* and *Sxl* mutant germlines cannot be feminized (Fig. 1F,G). However, the *hs-otu* construct did cause an increase in the number of gonads possessing VASA-positive cells (compare the non-oogenic and atrophic classes in Fig. 1B,F). The proportion of non-oogenic *ovo* mutant pseudomale gonads increased from 18% without *hs-otu* to 64% with the construct, in effect equaling the frequency of non-atrophic gonads in ovo^{+} pseudomales (Fig. 1A). A more modest improvement was also obtained in *Sxl* mutant *XX* pseudomales, where the frequency of VASA-positive gonads increased from 52% to 74% (Fig. 1D,G). These results indicate an additional role for *otu* in some process affecting germline viability and/or proliferation that is separable from oogenic differentiation and independent of *ovo* and, possibly, *Sxl* functions.

The *otu* and *ovo* promoters are differentially regulated

The finding that *hs-otu* can feminize *XX* pseudomale germ cells suggests oogenesis is blocked because of insufficient *otu* levels. We therefore examined whether *tra*-induced sexual transformation affects the level of *otu* gene expression. In addition, we compared the regulation of the *ovo* and *otu* promoters to confirm our previous suggestion that these two genes are differentially regulated (Nagoshi et al., 1995) and to investigate the possibility that *ovo* may control *otu* promoter activity (Lu et al., 1997).

Two constructs were utilized in which the *otu* (*otu-lacZ*, Rodesch et al., 1995) or *ovo* (*ovo-lacZ*, Mèvel-Ninio et al., 1995) promoters were fused to the structural *lacZ* gene. We first compared their developmental expression patterns in wild-type gonads. Both were expressed specifically in the adult female germline, but differed in their activity during preadult stages. *otu-lacZ* was expressed in most, if not all, larval and pupal germ cells in both female (Rodesch et al., 1995) and male gonads (Fig. 4A,B). Sex-specific regulation only became apparent in the adult testis where male germline expression became restricted to a few cells at the apical tip (Fig. 4C; see also Hager and Cline, 1997 for analogous results with a similar construct). As with *otu*, the *ovo* promoter is initially active in both male and female larval gonads (Mèvel-Ninio et al., 1995). However, *ovo-lacZ* becomes sex-specific at an earlier stage, showing restricted expression in male gonads during the third instar larval and pupal periods (Mèvel-Ninio et al., 1995; Figs. 4D,E). The expression pattern in the adult testis is identical to that seen with *otu-lacZ* and has been published elsewhere (Oliver et al., 1994). These results demonstrate that the *otu* and *ovo* promoters are under different regulatory control in the pre-adult germline.

otu, but not *ovo*, promoter activity is influenced by *tra*-induced sexual transformation

We next examined how changes in the somatic sex influenced *otu-lacZ* and *ovo-lacZ* activities. *otu-lacZ* expression was greatly reduced in *XX* pseudomales (data not shown); however, the interpretation of this result was complicated by the high frequency of degenerating germ cells in these gonads. To compensate for this problem, *otu-lacZ* was assayed in *hs-otu XX* pseudomale gonads which typically contained viable, oogenic germ cells (Fig. 1E). We found that even under these optimized conditions, *otu-lacZ* activity was

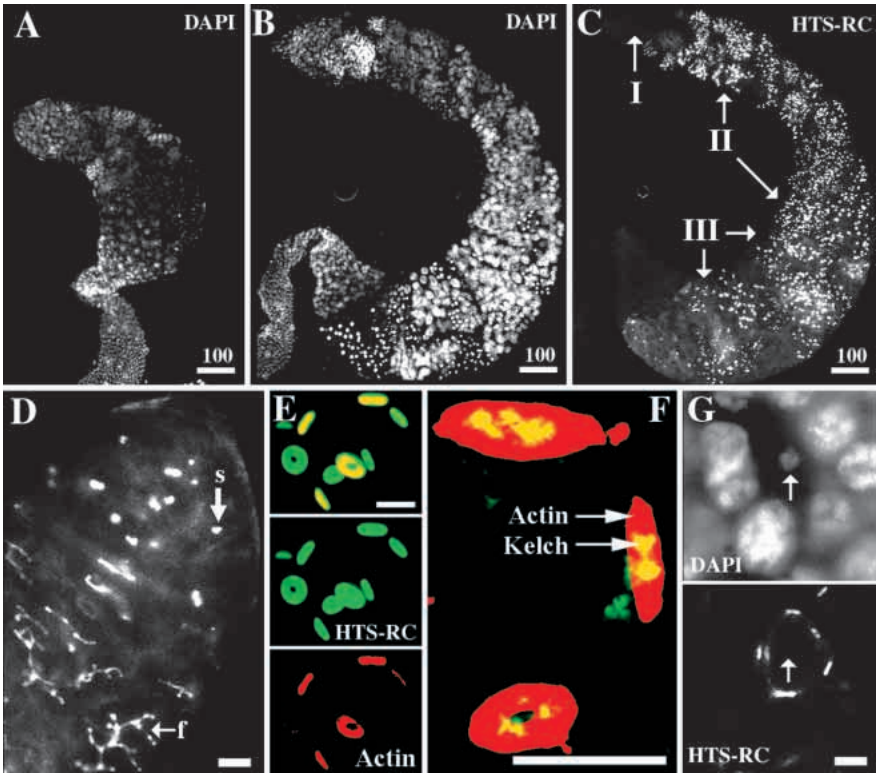

Fig. 3. Oogenic stages observed in *XX* pseudomale gonads carrying one copy of *hs-otu*. (A) *XX* pseudomale testis without *hs-otu* labeled with DAPI to label nuclei. (B) Testis of *XX* pseudomale carrying one copy of *hs-otu* labeled with DAPI. (C) *hs-otu* pseudomale testis labeled with anti-HTS-RC. A number of germ cell clusters have ring canals with HTS-RC. Sections I, II and III identify domains of germ cells at distinct developmental stages. (D) Confocal image of germ cells in Section I labeled with anti-spectrin antibodies. Spectrosomes (s) and fusomes (f) are detected. (E) Confocal image of ring canals located in Section II labeled with anti-HTS-RC (green) and phalloidin (red). Regions of overlap are in yellow (top plate). Lower two plates represent the individual HTS-RC channel and phalloidin channel, respectively. Note that not all rings have incorporated f-actin. (F) Confocal image of ring canals located in section III labeled with anti-KELCH (green) and phalloidin (red). Regions of overlap are in yellow. KELCH is localized in inner surface of f-actin layer. (G) Cells located in section III stained with DAPI (top) and anti-HTS-RC (bottom). DAPI channel shows large nurse-like cells surrounding small oocyte-like nucleus (arrow). HTS-RC channel showing five ring canals surrounding the oocyte nucleus (arrow). For D-G, the size bar is equal to 5 μm.

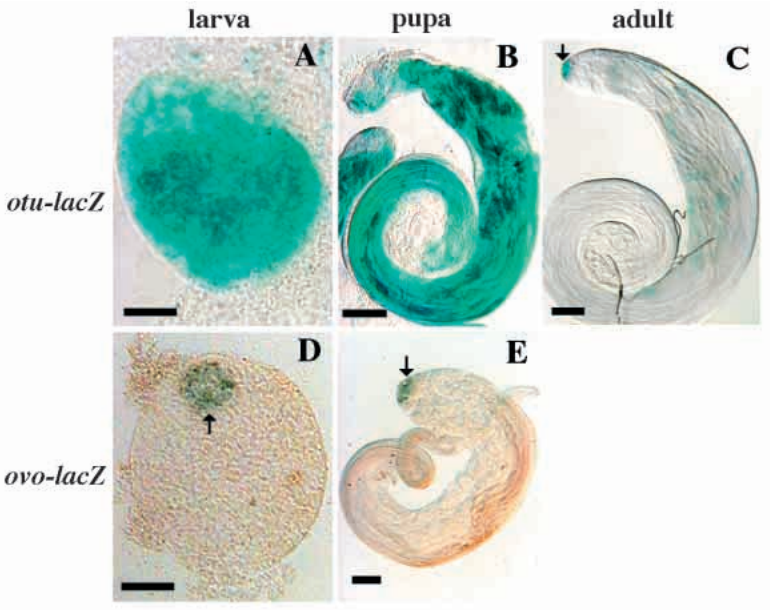

Fig. 4. *otu* and *ovo* promoter expression in wild-type animals. Animals carrying one copy of *otu-lacZ* or *ovo-lacZ* were stained for β-galactosidase expression (green stain). *otu-lacZ* expression in (A) a third instar larval testis, (B) a pupal testis and (C) an adult testis (expression is restricted to a few apical cells, arrow). *ovo-lacZ* expression in (D) a third instar larval testis (expression is restricted to only a few cells at most anterior region, arrow), and (E) a pupal testis (expression is still restricted to only a few apical cells, arrow). Size bar equals 100 μm.

reduced and sporadic, with β-galactosidase staining in a few isolated cell clusters in only a minority (3/13) of gonads (Fig. 5A,B). Even germ cells with clear oogenic phenotypes (such as large, nurse-like nuclei) typically showed little *otu-lacZ* expression. In contrast, *ovo-lacZ* was extensively expressed in all (10/10) *hs-otu XX* pseudomale gonads (Fig. 5C), consistent with previous studies demonstrating *ovo* promoter activity in *XX* pseudomales using a similar construct (Oliver et al., 1994). The same result was seen with a germline-specific enhancer-trap line, *BC69*, in which *lacZ* is controlled by the *vasa* promoter (Rodesch et al., 1995). All (14/14) *hs-otu XX* pseudomale gonads carrying *BC69* showed substantial expression of β-galactosidase (Fig. 5D). These data demonstrate that the *tra*-induced sexual transformation specifically inhibits *otu* promoter activity.

We also performed the reciprocal experiment in which *otu-lacZ* activity was examined in *XY* germ cells developing in a female somatic background. *XY* pseudofemales produced by the ectopic expression of *tra* result in ovaries containing tumorous egg chambers (McKeown et al., 1988). Because *XY* pseudofemale germ cells become sufficiently 'feminized' that they acquire a need for *otu* function for optimal proliferation (Nagoshi et al., 1995), we anticipated they would also be permissive for *otu* promoter activity. This was in fact the case. Even in the absence of *ovo* function, *XY* pseudofemale germ cells consistently expressed *otu-lacZ* (Fig. 5E). This indicates that the feminizing effects of *tra*, but not *ovo*, are necessary for *otu* transcription in this genotype. In comparison, the *ovo* promoter is not detectably active in *XY*

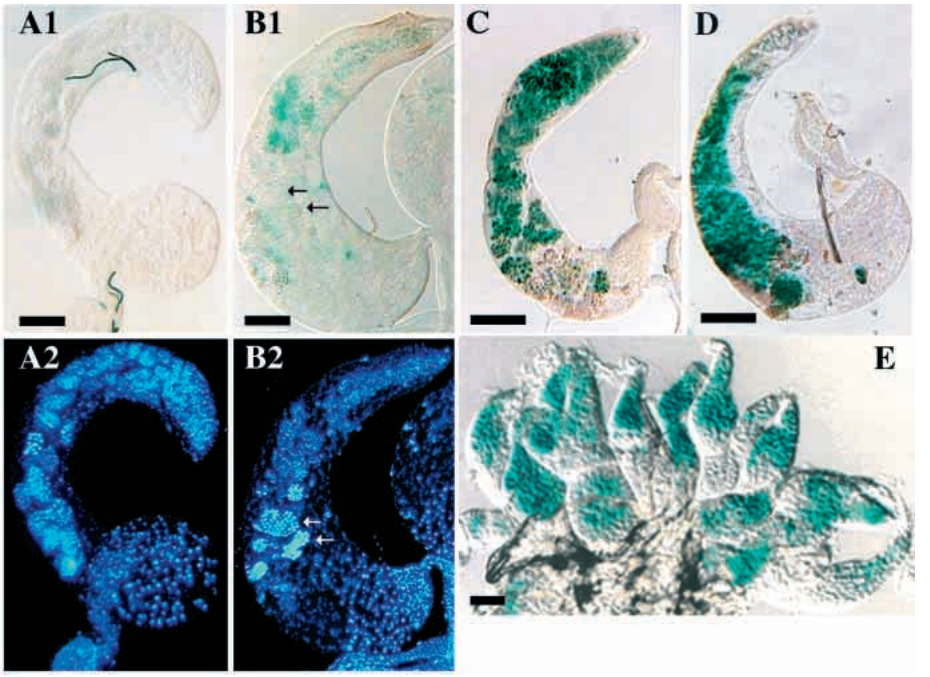

Fig. 5. *otu* promoter expression in sexually transformed animals. Adult *XX* pseudomale testes stained for β-galactosidase (green stain) and DAPI (β-galactosidase staining tends to suppress DAPI fluorescence). (A,B) *XX* pseudomales whose germlines were feminized by one copy of *hs-otu*. (A1) No β-galactosidase expression is observed although DAPI staining (A2) detects many germ cell clusters. (B1) Same genotype as A showing a subset of germ cells expressing β-galactosidase. Most cells do not express β-galactosidase , even those with large, nurse-like nuclei (B2, arrow). (C) *ovo-lacZ* expression in an *XX* pseudomale gonad. Unlike *otu-lacZ*, *ovo* is expressed in the majority of germ cells. (D) *XX* pseudomale carrying the *BC69* enhancer trap. Most germ cell clusters express β-galactosidase. (E) *otu-lacZ* expression (one copy) in ovo^{D1rS1}/Y pseudofemale somatically feminized by *hs-tra*. Even in the absence of *ovo*, the *otu* promoter is highly active. Size bar equals 100 μm.

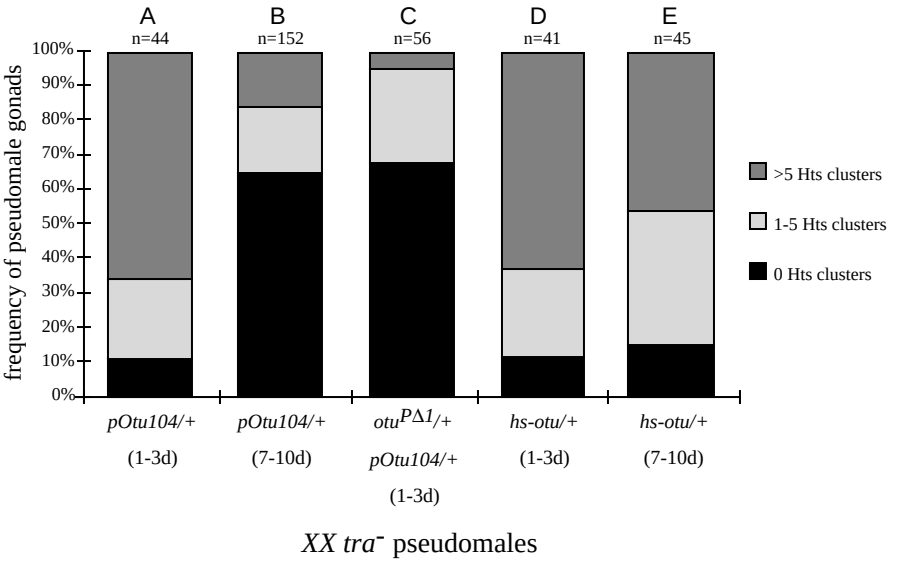

Fig. 6. Frequency of HTS-RC-expressing gonads in differentially aged *XX* pseudomales. *tra* mutant *XX* gonads were labeled with antibodies specific for HTS-RC. Genotypes from A-C are derived from the cross $otu^{P\Delta1}$ *v f/FM6*; tra^{4} kar^{2} ry^{5} *red/TM3* X *pOtu104/pOtu104*; tra^{1}/*TM6*. (A, B) *+/FM6*; *pOtu104/+*; tra^{4}/tra^{1}. (C) $otu^{P\Delta1}$/+; *pOtu104/+*; tra^{4}/tra^{1}. (D, E) *hs-otu/+*; tra^{4}/tra^{1} from the cross: *hs-otu/hs-otu*; tra^{4} *kar ry red/TM6* X *+/*$B^{s}Y$; tra^{1}/*TM6*. 1-3d, aged 1-3 days posteclosion before dissection; 7-10d, aged 7-10 days posteclosion; n, number of gonads examined for that genotype.

pseudofemales (Oliver et al., 1994), again illustrating differential regulation of *ovo* and *otu*.

We wanted to confirm that the somatic regulation of adult *otu* promoter activity could account for the pseudomale phenotypes. This was accomplished with the *pOtu104* construct, in which a functional *otu* cDNA is fused to *otu* promoter sequences. One copy of this transgene can complement to fertility *otu* null mutations (for description of an analogous construct see Sass et al., 1995). *XX* pseudomales were constructed with one copy of *pOtu104*, thereby carrying three doses of *otu* (two endogenous genes and the transgene). When dissected 1-3 days posteclosion, almost 90% of the gonads were oogenic (Fig. 6A) with the majority containing greater than five HTS-RC clusters. In contrast, eliminating one endogenous otu^{+} gene blocked this feminization (Fig. 6C). Therefore, three doses of *otu* genes or transgenes produce enough additional *otu* function, presumably during pupal stages, to induce oogenic differentiation. However, the level of feminization was markedly reduced if assayed 7-10 days posteclosion (Fig. 6B). This is consistent with the *otu* promoter (in the endogenous genes and *pOtu104*) becoming inactive shortly after eclosion. Supporting this interpretation, we found the feminization induced by the *hs-otu* construct was not substantially altered in similarly aged flies (Fig. 6D,E). In this case, the heterologous heat-shock promoter would not be inhibited by an *otu*-specific somatic interaction, hence oogenic differentiation should be maintained. Therefore, we conclude that *tra*-dependent female differentiation of the soma is necessary to maintain *otu* promoter activity in the adult germline at levels sufficient to support oogenesis.

DISCUSSION

Soma-dependent maintenance of *otu* activity in the *XX* germline is necessary for oogenesis

We previously demonstrated that female somatic signals can cause *XY* germ cells to require *otu* for proliferation (Nagoshi et al., 1995). This was the first indication that the germline function of *otu* was influenced by the sexual identity of the soma. We now extend these findings to show that a separable *otu* function controlling oogenic differentiation is also affected by somatic interactions. The heterologous expression of *otu* feminizes the *XX* pseudomale gonad at two levels. First, *hs-otu* expression dramatically increases the number of viable, differentiating germ cells as seen by the absence of atrophic gonads (Fig. 1E). Second, the germline can differentiate to more mature oogenic stages than typically seen in *XX* pseudomales, following the same progression of fusome and ring canal maturation events as occurs in normal oogenesis. In fact, the *hs-otu XX* pseudomale gonad becomes organized much like a normal ovarian germarium.

These results demonstrate a critical and rate-limiting role for *otu* in the somatic regulation of oogenesis. In effect, the developmental program for oogenic differentiation to about stage 4 remains intact in *tra* mutant *XX* pseudomale germ cells, but is usually inactive specifically because of inadequate levels of *otu* function. This is illustrated by our observation that *otu*-induced oogenesis cannot occur in the absence *ovo* or *Sxl*

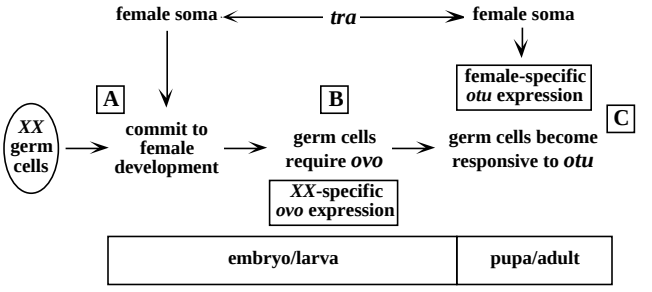

Fig. 7. Model for how *tra* influences the regulation and function of *ovo* and *otu*. *XX* germ cells initiate female development (A) in a process influenced by sex-specific somatic signals controlled by *tra*. Female differentiation of *XX* germ cells results in a requirement for *ovo* function during larval gonial stages (B). At about this stage, *ovo* promoter activity becomes restricted to *XX* germ cells. The function of *ovo* is required for germ cells to become responsive to the oogenic differentiation function of *otu* (C), beginning in pupal stages. At this time, sex-specific somatic signals controlled by *tra* induce female-specific *otu* promoter activity.

functions (Fig. 1C,D). Therefore, both these and other essential gene products must either be present in pseudomales or induced by additional *otu* activity. We conclude that the *tra*-dependent somatic interaction required for early stages of oogenesis acts primarily by modulating *otu* expression.

tra is required to maintain *otu*, but not *ovo*, promoter activity in the *XX* germline

The mechanism by which the soma influences *otu* function is through the regulation of the *otu* promoter. The male transformation of *XX* soma inhibits *otu* activity in *XX* germ cells, while the presence of pseudofemale soma allows *otu* expression in *XY* germ cells. This pattern of soma-dependent regulation is specific to *otu*, as two other germline promoters (*vasa* and *ovo*) were not affected by the same somatic sex transformations.

Further comparisons between the *otu* and *ovo* promoters are instructive. Both the *otu-lacZ* and *ovo-lacZ* promoter constructs initially have non-sex-specific expression patterns that subsequently become very restricted in the male germline (Mèvel-Ninio et al., 1995; Rodesch et al., 1995; Fig. 4). Although the potential perdurance of the β-galactosidase products preclude the precise determination of when sex-specific regulation begins, it is clear that it occurs earlier for *ovo* than for *otu*. Sex-specificity in the *ovo-lacZ* pattern is seen in 2nd and 3rd instar larval gonads (Mèvel-Ninio et al., 1995), compared to adult stages for *otu-lacZ* (Fig. 4). It is notable that these time periods approximately correspond to when the *ovo* and *otu* mutant germline phenotypes first become apparent (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995).

These observations demonstrate that sex-specific promoter activity for both genes is not initially determined by the germline X:A ratio. Instead, there is some type of developmentally regulated induction of sex-specificity, which occurs at different times for the two promoters. The regulation of *ovo* and *otu* transcription also differ in their sensitivity to somatic influences. Changes in the somatic sexual identity by alterations in *tra* expression have no effect on the activity of the *ovo* promoter (Oliver et al., 1994), but do alter *otu* promoter expression (Fig. 5A,B). Therefore, different mechanisms regulate the transcription of these two genes. In addition, *otu* promoter activity does not require *ovo* function in either *XX* or *XY* germ cells during early gametogenic stages (Rodesch et al., 1995; Fig. 5E). These data have important implications with respect to recent reports suggesting *otu* transcription is controlled by *ovo* (Lu et al., 1998). Our results indicate that this putative regulation does not play a major role during early stages of female germline development. Perhaps it becomes more critical during later oogenic stages.

otu has *ovo*-dependent and independent functions

In *XX* ovaries, mutations in *otu* affect germ cell numbers as well as their differentiation, suggesting a possible proliferation function. Support for this came from our earlier study using sexually transformed *XY* animals, which allowed us to separately examine the proliferation and differentiation phenotypes (Nagoshi et al., 1995). In this case, *XY* germ cells developing in a somatic ovary (*XY* pseudofemales) required *otu* to maintain germ cell and egg chamber numbers. We now demonstrate in pseudomales that a similar effect occurs in *XX* germ cells. The *ovo* null mutation prevents oogenic differentiation in *XX* pseudomales, even with *hs-otu* (Fig. 1B). However, the increased *otu* expression substantially improved the recovery of non-oogenic gonads (compare Fig. 1B with F). Therefore, *otu* appears to improve either the viability or proliferation of *ovo* mutant germ cells independent of further oogenic differentiation.

These observations have an additional ramification. In contrast to the *otu* proliferation function, the capacity for *otu* to induce oogenesis clearly requires *ovo* product (compare Fig. 1E with 1F). This dichotomy in the responsiveness of *ovo* mutant germ cells to *otu* activity indicates differences in how these two genes interact in regard to maintaining *XX* germ cell numbers and oogenic differentiation.

The genetic basis of the *XX* pseudomale germline phenotypes

From the data in Fig. 1, some inferences can be made concerning the genetic derivation of the pseudomale phenotypes. In every genotype examined, at least 10-20% of the pseudomales were of the non-oogenic class, even in those gonads containing *hs-otu* (Fig. 1). We believe this represents an early commitment of the germ cells in this subset to a male fate, as suggested by their fusome composition and operationally defined by their insensitivity to changes in the level of *otu* and mutations in *ovo* and *Sxl*. In this regard, they are similar to *XY* germ cells developing in testes which also do not require the functions of these oogenic genes. These data are consistent with previous studies based on morphological criteria that also identified a subset of 'spermatogenic' *tra* mutant pseudomales (Nöthiger et al., 1989). The implication is that a *tra*-dependent somatic process can sexually transform *XX* germ cells, albeit inefficiently, such that they no longer require *ovo*, *otu* or *Sxl* for at least some aspects of gametogenesis.

In the remaining oogenic and atrophic gonads, we believe the germ cells maintain a female identity, reflecting their chromosomal constitution. This 'femaleness' is defined by the continued sensitivity of these cells to the oogenic effects of heterologous *otu* expression (compare Fig. 1A and E). The degree of female development, and thereby the distinction between atrophic and oogenic gonads, depends upon the level of *otu* expressed, as demonstrated in Fig. 6.

tra-dependent somatic influences on *ovo* and *otu* germline functions

Mutations in the *ovo* gene typically result in the absence of germ cells in adult ovaries and aborted oogenic differentiation. In fact, *ovo* null *XX* germ cells were reported to die beginning in embryonic stages (Oliver et al., 1987, 1990). This effect on both the differentiation and early viability of the *XX* germline has led to the proposal that *ovo* is a primary regulator of germline sex determination, perhaps having a role in germline dosage compensation or in determining the germ cell X:A ratio (Oliver et al., 1990, 1993, 1994).

One assumption of these hypotheses is that the reduction in the number of *ovo* mutant germ cells is due to increased lethality. Evidence in support of this supposition comes from electron microscopic examination of presumptive *ovo* mutant embryos, where a subset of pole cells appeared to be dead or dying (Oliver et al., 1990). However, two separate studies demonstrate that consistent and significant abnormalities in *ovo* mutant gonads were not evident until the second instar

larval stage, bringing into question the significance of the reported embryonic phenotype (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). In neither of these later reports was there evidence that the larval gonadal abnormalities stemmed from germ cell lethality, as opposed to, for example, reduced cell proliferation. Therefore, it is not established that *ovo* is required for germ cell viability during oogonial development.

A second assumption is that the requirement for *ovo* is determined primarily, if not solely, by the germline X:A ratio. Support for this comes from two complementary observations: *XY* germ cells do not require *ovo* when made to develop in a female soma (Steinmann-Zwicky et al., 1989; Oliver et al., 1994), and the presence of male-transformed soma did not eliminate the requirement for *ovo* in *XX* germ cells (Steinmann-Zwicky et al., 1989; Nagoshi et al., 1995). However our use of molecular germline markers defined a more complex pseudomale phenotype. While we confirmed that an *ovo* null allele did prevent oogenic differentiation, no significant alterations in the frequency of the non-oogenic pseudomale class were observed. Therefore, the pseudomale transformation allows a substantial fraction of *ovo* null *XX* germ cells to perdure to the adult stage and produce spectrosomes and fusomes. This contrasts with the absence of similar differentiation typically observed in the infrequent *ovo* null germ cells found in adult ovaries (Fig. 2), although it should be noted that a small subset of these mutant cells can occasionally produce fusomes (R. N. N. and S. H., unpublished data). A similar phenomenon is seen with *otu*. In females, null mutations in *otu* block oogenesis at the first cystocyte division, producing small, linear fusomes (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). In contrast, non-oogenic pseudomales lacking *otu* produce large, multibranched fusomes (data not shown). We therefore conclude that the requirements of *XX* germ cells for *ovo* and *otu* functions, at least with regard to early gametogenic differentiation, can be substantially influenced by *tra*-dependent changes in the sexual state of the soma.

One explanation for these results follows from our genetic rationalization of the pseudomale phenotypes. We hypothesize that, despite their female chromosomal constitution, *XX* germ cells committed to a male fate do not need *ovo* or *otu* to initiate gametogenesis. In one simple permutation of this model, the sexual identity of the germline is defined prior to *ovo* and *otu* functions by a process involving *tra*-dependent somatic interactions. In this case, *XX* pseudomale germ cells committed to a male fate initiate spermatogenesis independent of *ovo* and *otu*. In contrast, those that retain a female identity still require these genes and so become atrophic in their absence.

This early placement of a somatic interaction contributing to germline sex determination is consistent with studies indicating that *tra* influences the development of the *XX* germline during embryonic and early larval stages (Seidel, 1963; Steinmann-Zwicky, 1994a; Staab et al., 1996), while *ovo* and *otu* appear to be required later in development (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). However, in this regard, it should be noted that there is a significant maternal contribution of *ovo* product, which could mask possible effects of *ovo* mutations during early stages of germline differentiation (Mèvel-Ninio et al., 1995).

To summarize our results, we suggest a model describing the regulatory relationships between *ovo*, *otu* and *tra*. We propose that a somatic signal controlled by *tra* contributes to an early regulatory commitment to female development (Fig. 7A). This creates a requirement for *ovo* activity during the larval gonial stages, at which time the *ovo* promoter becomes more sex specific in its expression (Fig. 7B). We believe that during the pupal and adult stages, two critical events occur in the female germarium (Fig. 7C). First, *ovo* activity allows *XX* germ cells to become receptive to the *otu* function controlling oogenic differentiation. Second, *tra*-dependent somatic signals allow continued expression of *otu* in the female germline by maintaining *otu* promoter activity. By this mechanism, we believe that the *otu* gene serves to link the somatic sex differentiation pathway controlled by *tra* with a female germline developmental pathway controlled by *ovo*.

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